Virology 273, 325–332 (2000)
doi:10.1006/viro.2000.0437, available online at http://www.idealibrary.com on IDEAL®

Cloning of an Infectious Milk-Borne Mouse Mammary Tumor Virus (MMTV) DNA from a Mammary Tumor That Developed in an Endogenous MMTV-Free Wild Mouse

Lai Xu,*,† Chin H. Tay,† Brigitte T. Huber,† and Nurul H. Sarkar*,[1]

*Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, Georgia 30912; and
†Department of Pathology, Tufts University School of Medicine, Boston, Massachusetts 02111

Received January 6, 2000; returned to author for revision April 6, 2000; accepted May 24, 2000

Molecular characterization of infectious mouse mammary tumor viruses (MMTVs) has been hampered due to the problem of cloning a full-length exogenous virus into a plasmid. The present report describes our strategy for obtaining a full-length clone of an exogenous MMTV from a mouse mammary tumor that arose spontaneously in a wild Chinese mouse free of endogenous MMTV and shows that the cloned virus (JYG-MMTV) is expressed in rat RBA cells. Four-week-old C58/J × CBA/CaJ female mice, free of both endogenous and exogenous MMTVs, were injected with virus-secreting RBA cells. The progeny of these mice were bred, and their offspring were tested for the presence of MMTV. These third-generation mice were found to actively produce MMTV that was shed in their milk and transmitted to their offspring. The virus was detected not only in the mammary glands of these young mice, but also in their spleens and bone marrow. These results suggest that our plasmid-cloned exogeneous JYG-MMTV is infectious. This virus can now be used effectively in manipulating the various genes of JYG-MMTV and other MMTV strains to understand their structure/function relationships.

INTRODUCTION

Mouse mammary tumor virus (MMTV), a B-type retrovirus, was first identified in 1936 as a "milk factor" in certain strains of mice with a high incidence of breast cancer (Bittner, 1936). It is now known that there are two forms of MMTVs: exogeneous MMTVs are transmitted from mother to the offspring through milk, whereas the endogenous MMTVs, the majority of which do not produce infectious viral particles, are proviral integrants and are transmitted through the germ line (Kozak *et al.,* 1987). One recent study has indicated the generation *in vivo* of infectious MMTVs by recombination between endogenous and exogenous proviruses (Golovkina *et al.,* 1997). The MMTV provirus is composed of *gag, pro, pol,* and *env* genes and serves as a template for three viral mRNA transcripts: genomic, envelope (*env*), and long terminal repeat (LTR) mRNAs (Sen *et al.,* 1979, 1980; Wheeler *et al.,* 1983; Van Ooyen *et al.,* 1983). Two copies of the viral RNA genome are packaged into viral particles which are released from mammary cells and shed into the milk. The viral LTR mRNA translates into a membrane-associated glycoprotein that has been designated a superantigen (*SAg*) since it leads to *in vivo* deletion and *in vitro* stimulation of T cells bearing particular Vβ gene products (Acha-Orbea *et al.,* 1991; Choi *et al.,* 1991; Frankel *et al.,* 1991; Held *et al.,* 1992). *SAg* seems to play an important role in MMTV pathogenesis (Dzuris *et al.,* 1997; Golovkina *et al.,* 1992; Held *et al.,* 1992, 1993).

Mouse mammary tumors develop as a consequence of the integration of infectious MMTVs next to a family of protooncogenes such as *int*-1, *int*-2, and *int*-3, which can be transcriptionally activated by the integrated virus (Dickson *et al.,* 1984; Gallahan and Callahan, 1987; Nusse and Varmus, 1982; Nusse *et al.,* 1984; Peters *et al.,* 1983; Sarkar *et al.,* 1994). These protooncogenes are also involved in the control of developmental processes in vertebrates (Nusse and Varmus, 1992). The expression of one of the *int* genes, *int*-3, has been shown to be increased dramatically by steroid hormones (Robbins *et al.,* 1992) that act on the inducible enhancer contained within the viral LTR (Ostrowski *et al.,* 1984; Ucker *et al.,* 1981).

To date, structural and functional studies of exogenous MMTVs have been very difficult. Although the full-length proviral DNA of an exogenous MMTV, GR-MMTV, was successfully cloned into λ phage, this clone was found to be unstable during phage DNA replication (Morris *et al.,* 1989). Plasmid cloning of full-length exogenous BR6-, GR-, and C3H-MMTV has been obstructed by two barriers (Brookes *et al.,* 1986; Donehower *et al.,* 1981; Fasel *et al.,* 1983; Groner *et al.,* 1980; Majors and Varmus, 1981, 1983; Morris *et al.,* 1989; Peterson *et al.,* 1985; Salmons *et al.,* 1985; Shackleford and Varmus, 1988; Xu *et al.,* 1994). First, a "poison" sequence contained within the *gag* gene prevents the *gag* gene from being cloned into plasmids. The second barrier is that the two LTRs of the MMTV provirus have a tendency to be deleted when

[1] To whom reprint requests should be addressed. Fax: 706-721-7915. E-mail: nsarkar@mail.mcg.edu.

0042-6822/00 $35.00

the proviral DNA is amplified in *Escherichia coli.* However, it has been possible to construct a hybrid MMTV (Hyb-MMTV) containing the 5′ segment of an endogenous MMTV, the *Mtv*-1, and the 3′ segment of an exogenous C3H-MMTV (Shackleford and Varmus, 1988). This Hyb-MMTV is able to induce mammary tumors in BALB/c mice that carry three endogenous MMTVs, *Mtv*-6, *Mtv*-8, and *Mtv*-9. However, the hybrid virus has recently been shown to undergo recombination with *Mtv*-8 and *Mtv*-9 for the development of mammary tumors in BALB/c mice (Wrona *et al.,* 1998). Reevaluation of the results that Shackleford and Varmus (1988) obtained with their Hyb-MMTV indicates that the tumors they analyzed were most likely induced by Hyb-MMTV/*Mtv*-9 and Hyb-MMTV/Mtv-8 recombinants, because of the fact that the tumor DNA contained amplified copies of a *Mtv*-9-specific 2.7-kb MMTV-*gag*-related *Pst*I/*Bgl*II fragment and a *Mtv*-8- and *Mtv*-9-specific 2.1-kb MMTV-*env*-related *Bgl*II fragment (see Fig. 6). These observations underscore the problem of using hybrid MMTVs in studies of the genetics of exogenous viruses in mouse strains carrying endogenous MMTVs.

We reported previously that a novel strain of feral mice (JYG) carrying only exogenous MMTV (JYG-MMTV) develop mammary tumors at a high incidence as a consequence of insertional mutations in *int*-3 (40%), *Wnt*-1 (33%), and *int*-2 (13%) (Imai *et al.,* 1994; Sarkar *et al.,* 1994). In contrast, GR- and C3H-MMTVs have been shown to activate only *Wnt*-1 and/or *int*-2, while CzechII-MMTV activates *int*-3 (Nusse and Varmus, 1982; Dickson *et al.,* 1984; Gallahan and Callahan, 1987). Furthermore, GR-MMTV and JYG-MMTV differ in host cell range: the GR-MMTV infects both ductal and lobuloalveolar mammary epithelial cells and induces type-B and -P adenocarcinomas (Van Nie and Dux, 1971; Sass and Dunn, 1979; Van Nie, 1981), whereas JYG-MMTV infects only lobuloalveolar cells and causes only type-B adenocarcinomas (Imai *et al.,* 1994). These differences in the patterns of MMTV oncogenesis regarding the activation of different oncogenes in different strains of mice may be reflected in the genetics of the various structural genes among the different exogenous MMTV strains as well as in the participation of endogenous MMTVs.

In order to understand the basis for the preferential infection and activation of *Wnt/int* genes by MMTV in different mouse strains, it is imperative that studies of the biology of the genes of the various exogenous MMTV strains be undertaken. The most effective approach for such analyses may involve (1) plasmid cloning of a particular infectious MMTV, such as JYG-MMTV, (2) construction of chimeric proviruses by switching gene fragments among different exogenous MMTV strains, (3) studies of the pattern of viral infection and mammary tumor development in endogenous MMTV-free mice, and finally (4) analyses of the pattern of *int* activation in the tumors. To facilitate such studies, we have cloned a full-length JYG-MMTV in a plasmid and show that the provirus is infectious.

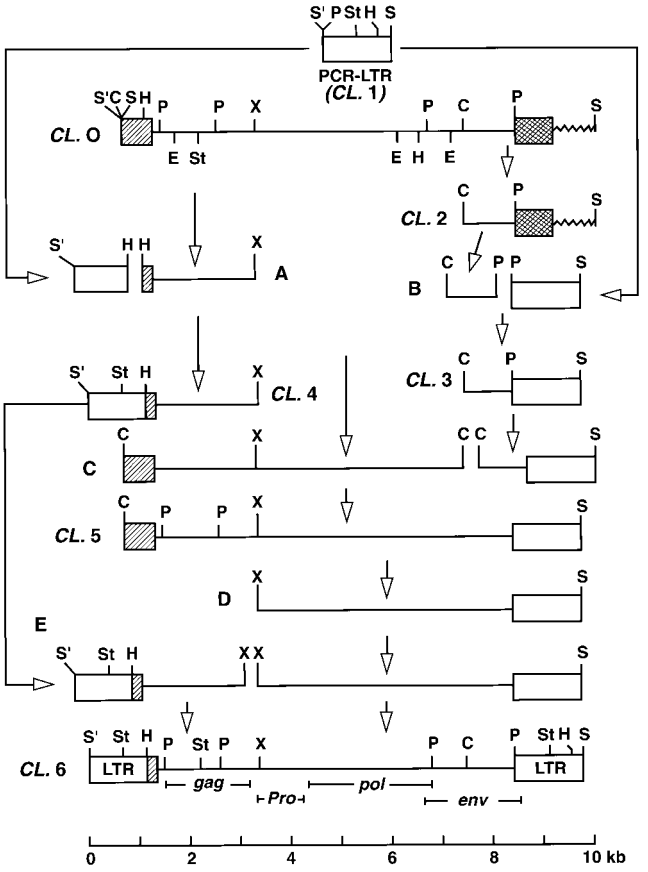

FIG. 1. Diagram depicting the steps used to construct a full-length JYG-MMTV provirus (*CL.* 6; for details see text) from a plasmid clone *CL.* O (previously designated as clone No. 11; Xu *et al.,* 1994) of the provirus that contained truncated long terminal repeats (LTRs). The truncated 5′ and 3′ LTRs in *CL.* O have been indicated by two hatched boxes; the clone contains about 0.9 kb cellular sequences at the 3′ end (indicated by a wavy line). The blank box represents a full-length LTR cloned in plasmid by PCR (*CL.* 1). Restriction sites for *Sal*I(S′), *Cla*I(C), *Sac*I(S), *Hin*dIII(H), *Pst*I (P), *Eco*RI(E), *Stu*I (St), and *Xba*I(X) in the provirus have been indicated (see *CL.* O and *CL.* 6). Note that the *Cla*I(C)/*Cla*I(C) fragment shown in C was derived from *CL.* O. The locations of the *gag, pro, pol,* and *env* genes of the full-length provirus are shown below *CL.* 6.

RESULTS

Reconstruction of a full-length JYG-MMTV provirus in pBluescript SK

Previously, we cloned a 9.4-kb JYG-MMTV provirus containing the 5′ LTR-*gag-pol-env*-3′ LTR into pBluescript SK (described as clone No. *CL.* 11 in Xu *et al.,* 1994). In the present report, this original clone has been designated as clone O (*CL.* O, Fig. 1; the plasmid segment of the construct is not shown). While this provirus contained the open reading frames for the *gag, pro, pol,* and *env* genes, both the 5′ and the 3′ LTRs (hatched boxes) were partially deleted; the 3′ end of the viral DNA

contained about 0.9 kb cellular sequences. Fortunately, the deleted LTRs were found to be overlapped by 8 bp. This allowed us to compile the sequence of a full-length LTR and to design primers for the PCR amplification of a full-length LTR fragment to replace the truncated LTRs contained in clone *CL.* O (Fig. 1). A full-length LTR was directly amplified from the genomic DNA of a JYG mouse mammary tumor to ensure that only the LTR of exogenous MMTV proviruses was amplified. The amplified 1.33-kb LTR product was digested with *Pst*I (P) and *Sac*I (S) and subcloned into pBluescript SK (*CL.* 1) and sequenced. No mutation was found to be present in the plasmid clone and, therefore, this clone was used to construct a full-length provirus following a number of steps (Fig. 1). It should be mentioned that our initial attempts at cloning this full-length LTR into the *gag-pol-env* fragment failed, as deletion of either the 5′ or the 3′ LTR occurred frequently when the plasmid was replicated in XL1-Blue *E. coli.* However, this problem was readily overcome by using SURE cells (Stratagene) as the bacterial host.

The plasmid clone (*CL.* O) containing a truncated JYG-MMTV proviral fragment (8.33 kb) was first digested with *Cla*I (C), and the *Cla*I-*env*-Δ 3′ LTR-cellular-pBluescript SK-*Cla*I fragment was isolated and then self-ligated (see Fig. 1: the pBluescript SK-*Cla*I site is located at the beginning of the 5′ ΔLTR). This clone (*CL.* 2) was then modified by substituting the 1.6-kb *Pst*I-Δ 3′ LTR-cellular DNA-*Sac*I fragment with a full-length LTR: the *Cla*I (C)-*env-Pst*I (P) segment from clone *CL.* 2 (Fig. 1B) was combined with the 1.33-kb *Pst*I-LTR-*Sac*I fragment from clone *CL.* 1. The resulting clone has been designated *CL.* 3. In order to restore the full-length 5′ LTR of the provirus, clone *CL.* 4 was constructed first by ligating the *Sal*I (S′)-ΔLTR-*Hin*dIII (H) fragment from *CL.* O and the *Hin*dIII-5′ ΔLTR-*gag-Xba*I (X) fragment from clone *CL.* O (Fig. 1A) into the *Sal*I/*Xba*I-digested pBluescript vector. The *Cla*I-Δ5′ LTR-*gag-pol-env-Cla*I fragment was then isolated from clone *CL.* O (Fig. 1C) and cloned into the *Cla*I site of clone *CL.* 3 to produce clone *CL.* 5. The truncated 5′ LTR contained in *CL.* 5 was then replaced with a full-length 5′ LTR. To accomplish this goal, clone *CL.* 5 was digested with *Xba*I and the *Xba*I-Δ*gag-pro-pol-env*-3′ LTR fragment (Fig. 1D) was isolated and ligated with the *Sal*I-5′ ΔLTR-*gag-Xba*I fragment that was obtained from clone *CL.* 4 (Fig. 1E; the *Sal*I site in clone *CL.* 4 is located within the plasmid). This final step resulted into the development of a plasmid clone (*CL.* 6) of a full-length JYG-MMTV provirus.

In order to determine whether the cloned provirus had undergone any rearrangements or deletions during reconstruction, DNA was digested with the restriction enzymes *Eco*RI (E), *Pst*I (P), *Stu*I (St), *Hin*dIII, *Sac*I (S), and *Sal*I (S′) and analyzed by Southern blotting and hybridization with full-length MMTV (MMTV-*rep;* Fig. 2B) and MMTV-LTR (Fig. 2C), and -*gag* probes (Fig. 2D). The

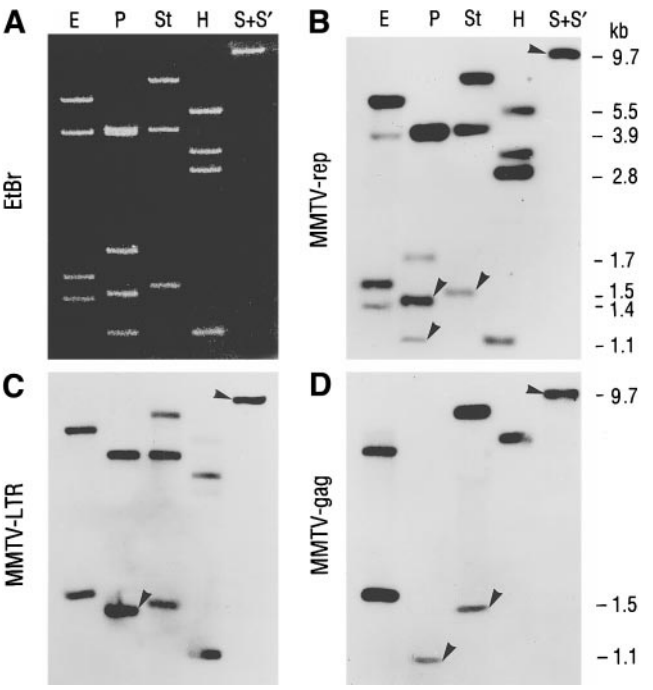

FIG. 2. Evidence that the reconstructed full-length JYG-MMTV provirus subcloned in the *Sal*I-*Sac*I sites of pBluescript SK is intact: the hybridization patterns are consistent with the restriction map of a full-length JYG-MMTV (see *CL.* 6 in Fig. 1). The proviral DNA was digested with *Eco*RI (E), *Pst*I (P), *Stu*I (St), *Hin*dIII (H), and *Sac*I-*Sal*I (S+S′) and then electrophoresed. The gel was stained with ethidium bromide (A) and subsequently hybridized with full-length MMTV (MMTV-rep; (B), MMTV-LTR (C), and MMTV-*gag* (D) probes. The fragments of defined sizes that were expected to be produced upon digestion of a full-length JYG-MMTV provirus by the restriction enzymes P, St, and S+S′ have been indicated by arrows. The diagnostic fragments are (1) a 1.4-kb fragment that includes 5′ LTR and sequences upstream to the first *Pst*I site (lane P; B and C); (2) a 1.1-kb *gag* (lane P; B and D); (3) a 1.5-kb 5′ LTR-*gag* (lane St; B, C, and D): and (4) a 9.7-kb provirus (lane S+S′; B, C, and D).

radioactive probes were prepared using an oligo-labeling kit (Pharmacia). The sizes of the restriction fragments visualized by ethidium bromide staining (Fig. 2A) and by the radioactive probes were found to be consistent with the restriction map of the full-length JYG-MMTV provirus (compare Figs. 1 and 2). This indicated that no rearrangements or deletions occurred within the reconstructed provirus. As a precaution, we subcloned again the 3′ LTR of the full-length provirus into pBluescript SK and its nucleotide sequences were determined. No point mutations were found in the LTR. Taken together, the results suggested that the cloned provirus was intact and both the 5′ and the 3′ LTRs remained unaltered during DNA amplification in SURE cells.

Evidence for the infectivity of the cloned provirus

MMTV is a slow-growing oncogenic virus and does not efficiently transform cells *in vitro.* Therefore, MMTV pathogenicity must be tested *in vivo.* To achieve this goal

for cloned MMTV proviruses, viral constructs are usually transfected into a suitable cell line, followed by intraperitoneal and/or subcutaneous injection of the virus-producing cells into susceptible mice (Shackleford and Varmus, 1988). One cell line, RBA (ATCC), derived from a rat mammary adenocarcinoma, has been shown to be suitable for MMTV proviral transfection since these cells have glucocorticoid receptors, but no MMTV-related sequences, in their genome. However, it is well known that cloned MMTVs do not express efficiently in transfected cells. To boost expression of a hybrid MMTV in transfected cells, Shackleford and Varmus (1988) introduced, upstream to the MMTV 5′ LTR, a 0.3-kb transcriptional enhancer obtained from Moloney murine leukemia virus (Mo-MLV). The placement of such an enhancer element upstream to an MMTV LTR construct has been shown to result in a 25-fold increase in expression of a reporter gene in the presence of dexamethasone (Ostrowski *et al.,* 1984). Taking these observations into account, we introduced a 0.3-kb DNA fragment containing the Mo-MLV enhancer into the JYG-MMTV provirus by linearizing the proviral construct with *Sal*I (*Sal*I site is located at the beginning of the 5′ LTR). The viral construct was analyzed (data not shown) and found to be suitable for transfecting RBA cells. Twenty colonies of stable transformants were initially picked for expansion and analyzed for the presence of MMTV proviruses by PCR (Fig. 3A) using LTR primers. Fifteen of the 20 cell lines were found to contain MMTV proviruses (data not shown). Although 7 of the MMTV-DNA-positive cell lines were found to produce MMTV particles, the results obtained with one cell line are shown in the present report.

To test whether the MMTV-DNA-positive RBA cells produced JYG-MMTV, culture medium was harvested from cells that were treated with 0.7 μM dexamethasone for 24 h and processed for virus isolation, followed by viral RNA extraction. RNA samples were then treated with DNAse in order to eliminate possible DNA contamination and subjected to DNA- and reverse transcriptase (RT)-PCR analysis using a set of LTR primers that were designed to amplify the sequences contained within the 3′ end of the viral genomic RNA, as well as the *SAg*-mRNA. DNA-PCR, done with RNA samples that were treated with DNase, did not yield any amplification product, whereas RT-PCR resulted in the production of a 0.8-kb band representing a 3′ segment of the viral genomic RNA (Fig. 3B). These observations thus indicated that some of our JYG-MMTV-transfected cell lines were producing MMTV particles.

In order to determine whether the particles produced by JYG-MMTV transfected cells were infectious *in vivo,* we used one JYG-MMTV-producing rat-RBA cell line directly to infect 4-week-old C58/J × CBA/CaJ female mice, free of both endogenous and exogenous MMTVs, and bred them (Scherer *et al.,* 1997). The first litter pups of these rat-cell-injected mice were discarded, but the pups

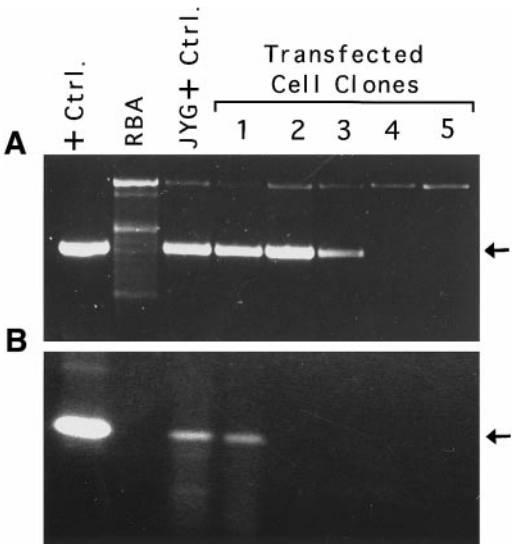

FIG. 3. A: Detection of 0.8-kb MMTV proviral DNA in JYG-MMTV (*CL*.6) transfected rat RBA Cells by genomic DNA-PCR. PCRs were carried out with a set of LTR-specific primers, spanning approximately 0.8 kb of the LTR, and 1 μg of cellular DNA. The PCR products were electrophoresed on a 1.0% agarose gel and stained with ethidium bromide. For positive controls, plasmid-cloned full-length JYG-MMTV DNA (+Ctrl) and DNA obtained from the tumor of a JYG mouse infected with JYG-MMTV (JYG+Ctrl) were used. DNA from nontransfected RBA cells was used as a negative control (RBA). Note that three of the five transfected cell lines shown in this illustration contained JYG-MMTV DNA. B: Detection of MMTV RNA in the medium of transfected rat RBA cells by RT-PCR. Culture medium from the transfected (clones 1–5) and nontransfected RBA cells (RBA) were collected, cellular contaminants were removed by low-speed centrifugation, and the supernatants were subjected to high-speed centrifugation. RNA was extracted from the pellets and used for cDNA synthesis. The cDNA was then amplified and the PCR products were electrophoresed on 1.0% agarose gel and stained with ethidium bromide. Two positive controls were used: the PCR product as used in A (+Ctrl) and RNA from a JYG-MMTV-producing mammary tumor cell line (JYG+Ctrl). RNA from nontransfected RBA cells were used as a negative control (RBA). Note that one of the transfected cell lines (clone 1) produced MMTV-RNA.

from the second litter (SL) were used for further breeding. This eliminated the possibility of MMTV-infected rat cells being transported to the offspring of SL mice. The lack of endogenous MMTV in C58/J × CBA/CaJ mice allowed the direct detection of exogenous MMTV genomes by PCR. Mammary glands, spleen, and bone marrow of young, third-generation, female SL mice were tested by DNA-PCR and RT-PCR for the presence of MMTV DNA and the expression of MMTV RNA, respectively. Mammary glands of lactating SL mice from the third generation were also analyzed for the presence of MMTV genomic and subgenomic mRNAs. Viral cDNA was made from total RNA. The DNA-PCR products were analyzed by Southern hybridization using an LTR-specific probe that was prepared by amplifying a 430-bp segment of JYG-MMTV LTR (LTR position: 470 to 902 bp). This internal fragment was chosen as a probe to avoid hybridization to the primers used for the DNA- and RT-PCR reactions. Southern blots of the PCR products obtained

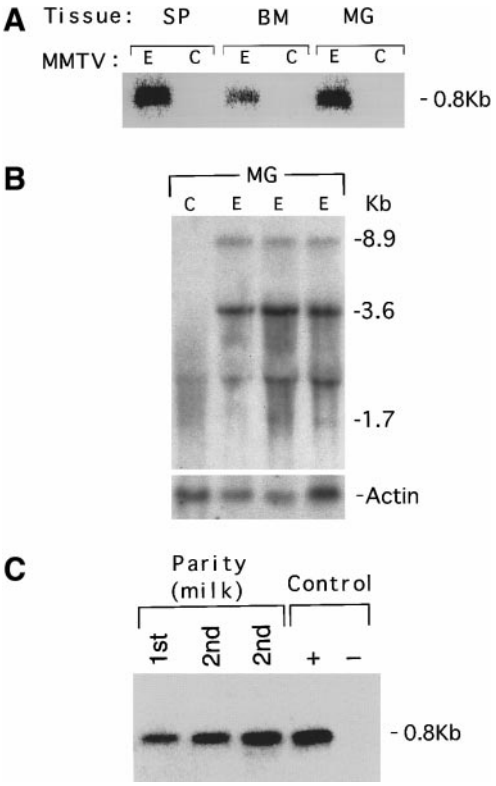

FIG. 4. Detection of 0.8-kb MMTV RT-PCR products in third-generation experimental (E) C58/J × CBA/CaJ mice (the founder animal did not carry any endogenous or exogenous MMTV). Total cellular RNA was prepared from spleen (SP), bone marrow (BM), and mammary glands (MG) of 2-week-old female mice. Note that the grandmother of these young animals was infected with cloned JYG-MMTV. Pooled tissue samples obtained from three animals were used for RNA extraction. PCRs were done with the 0.8-kb LTR-specific primers using 10 μg of cellular RNA. Tissues from uninfected mice were used as negative controls (C). The PCR products were electrophoresed on a 1.0% agarose gel and Southern blotted, and the filter was hybridized with a ^{32}P-dCTP-labeled 0.4-kb LTR probe. B: The upper panel shows the results of Northern blot analyses of total cellular RNA extracted from the lactating mammary glands of one control (lane 1) and three experimental (lanes 2–4) C58/J × CBA/CaJ mice. The grandmother of the third-parity experimental mice was infected with cloned full-length JYG-MMTV proviral DNA. Hybridization was performed with an MMTV-*rep* probe. The lower panel shows the result of hybridization of the same filter with an actin probe. C: Detection of 0.8-kb MMTV-RNA by Southern blotting of the RT-PCR products made from the milk of first- and second-parity mothers of third-generation of MMTV-infected C58/J × CBA/CaJ mice. Viral RNA was extracted from the milk and cDNA was synthesized and amplified by PCR with 0.8-kb LTR-specific primers. The PCR products were electrophoresed on a 1.0% agarose gel and Southern blotted and the filter was hybridized with a ^{32}P-dCTP-labeled 0.4-kb LTR probe. The positive (+) control PCR was performed with RNA isolated from the milk of an MMTV-producing JYG mouse, whereas the negative (−) control PCR was performed with RNA prepared from the milk of an MMTV-uninfected C58/J × CBA/CaJ mouse.

with the DNA (data not shown) and RNA (Fig. 4A) readily detected a 0.8-kb MMTV- specific band.

As shown in Fig. 4B, mice infected with cloned JYG-MMTV expressed the MMTV-specific 8.9-kb genomic RNA, the 3.6-kb *env*-mRNA, and the 1.7-kb LTR mRNA in their mammary glands. Milk collected from the stomachs of 2-day-old pups was also tested by RT-PCR for the presence of viral particles. The results presented in Fig. 4C demonstrate that MMTV was present in the milk of the virus-infected mice.

DISCUSSION

In this paper we describe for the first time the successful cloning of a full-length infectious provirus of JYG-MMTV. We achieved this goal by surmounting the two difficult cloning barriers that have hampered the study of the biology of MMTV. First, the difficulty in subcloning the *gag* gene, as shown by Brookes *et al.* (1986), was overcome (Xu *et al.,* 1994) by using the phagemid excision method widely used in cDNA cloning. The basic principle of phagemid excision is that DNA cloned into λZapII vector can be automatically excised with helper phage and recircularized to generate subclones in pBluescript phagemid (Short *et al.,* 1988; Sambrook *et al.,* 1989). Our present work demonstrates that the phagemid excision method allows the cloning into plasmid of an MMTV provirus that contains "toxic" sequences. Further, this method has recently allowed one of us (L. Xu) to subclone D^d (4.0 kb), a mouse MHC class I gene (unpublished results) that defied the conventional insert-ligating method due to the presence of some toxic sequences in its third exon (Evans *et al.,* 1982; Margulies, personal communication).

Regarding the second problem, our previous results clearly indicated that homologous recombinations between MMTV proviral LTRs can cause partial deletions of the viral genome, resulting in low recovery of full-length MMTV proviruses from a genomic DNA library (Xu *et al.,* 1994). As observed previously by many investigators, we also found pBluescript SK not to accept the provirus with intact 5′ and 3′ LTRs in certain bacterial hosts, such as XL1-Blue or JM109. The frequent occurrence of homologous recombinations between the 5′ and 3′ LTRs of GR-MMTV, possibly mediated by some bacterial DNA recombinases, has also been reported (Morris *et al.,* 1989). The use of SURE as the host cells avoided the problem of homologous recombination, since they are less permissive to homologous recombination because they lack *rec*B, *recJ*, *sbc*C, *umu*C, and *uvr*C activity. Thus, they can be used to clone unstable DNA structures, such as inverted repeats and Z-DNA (Stratagene).

As mentioned earlier, our restriction map of the JYG-MMTV provirus and the sequencing of the 3′ LTR suggested very strongly that our plasmid-cloned provirus is intact. Moreover, our biological study of JYG-MMTV demonstrated that the provirus-transfected RBA cells are able to produce virus and, more importantly, the virus thus produced is infectious to endogenous MMTV-free C58/J × CBA/CaJ F_2 mice.

Since the activation pattern of *Wnt/int* protooncogenes

is unique to JYG-MMTV (Sarkar *et al.,* 1994), it is possible that minor alterations in any of the structural genes of the JYG-MMTV and/or in the U3 region of its LTR, compared to other exogeneous MMTVs, may be responsible for the unique pattern of protooncogene activation in the mammary tumors of JYG mice. Furthermore, the differences in host cell range observed in various MMTVs may be due to differences among their *env* genes. These hypotheses can now be tested by making hybrid MMTVs by switching the LTR and/or the *env* segments between the JYG-MMTV and other exogenous MMTVs and comparing the patterns of the hybrid viruses to cell-specific infection and to the activation of *Wnt/int* in mammary tumors.

METHODS

DNA and RNA isolation

DNA was isolated from a primary mammary tumor (developed spontaneously in a JYG mice carrying only exogenous MMTV), mammary glands, and liver tissues of endogenous MMTV-free C58/J × CBA/CaJ F_2 mice of various generations, as well as normal RBA rat cells and the RBA cells that were transfected with a DNA construct containing a full-length copy of JYG MMTV DNA. RNA was also isolated from these tissues and cells, as well as from the supernatants of transfected cells. Genomic DNA and total cellular RNA were isolated following the protocols that we used previously (Xu *et al.,* 1994; Sarkar *et al.,* 1994). To obtain a sufficient amount of RNA from the mammary glands of young mice a pool of four glands was used.

Viral construct

To obtain a full-length copy of JYG-MMTV LTR, genomic DNA obtained from a mammary tumor of JYG mice infected with exogenous JYG-MMTV was subjected to PCR amplification. The following primers were used: the forward primer, 5′(6)-GCGCCTGCAGCAGAAATGGTTGAA(29)-3′, located at the 5′ end of the LTR, contained 25 U3-LTR-specific nucleotides with an internal *Pst*I site; and the reverse primer, 5′(1362)-GATCGAGCTCTGCCGCAGTCGGCGAAC(1345)-3′, located at the 3′ end of the LTR, contained 18 U5-LTR-specific nucleotides plus a *Sac*I linker, CACGTG, with 4 redundant nucleotides, GATC. Each 50-μl PCR reaction mixture contained 0.5 μg of the genomic DNA, 100 ng of each primer, and 1 U Taq polymerase (Promega). The reaction condition was 10 mM Tris–HCl (pH 9.0), 50 mM NaCl, 1 mM $MgCl_2$, 200 μM dATP, dCTP, dGTP, dTTP in a 50-μl reaction. Cycle times were 1 min at 95°C, 2 min at 55°C, and 1 min at 72°C. The PCR reactions were carried out using a Perkin–Elmer Cetus (Norwalk, CT) PCR DNA thermocycler. Thirty cycles were performed. The amplified LTR product (1.33 kb) was double digested with *Pst*I and *Sac*I, gel purified, and cloned into pBluescript following the method that we described previously (Xu *et al.,* 1994). To ensure that this PCR-generated LTR clone (Fig. 1, open boxes) did not suffer from possible deletion of some nucleotides or any rearrangement, the LTR was sequenced using the Sequenase Version 2.0 protocol from United States Biochemical. Single-strand sequencing was initiated with the pUC/M13 universal primer and continued with LTR-specific primers that we used in our previous work (Xu *et al.,* 1994). As this LTR was found to be intact, it was used in constructing a full-length JYG-MMTV (Fig. 1). A 0.3-kb DNA fragment containing the enhancer element of murine sarcoma virus was also ligated to the provirus following the strategy used by Shackleford and Varmus (1988).

Cell transfection

The cloned JYG-MMTV plasmid was used in transfecting RBA cells grown to 50–95% confluency in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Transfection was carried out at room temperature with 15 μg of the MMTV DNA and 1 μg pPuro DNA by electroporation at 320 V, 960 μF (Gene Pulser, Bio-Rad). The cuvettes were then transferred to an ice bath for 10 min and the sample gently transferred to six-well plates (Falcon, Lincoln Park, NJ). Stable transformants were selected with puromycin (3 μg/ml; Gibco/BRL). The presence of MMTV sequences in the transfected cells was examined by PCR using a second set of LTR primers: forward primer, 5′(209)-GCAGGGCTCTCTCTCACA(226)-3′; reverse primer, 5′(997)-TGTTTAGAGGGGACAGTTTTTA(976)-3′.

Virus harvest

The MMTV-transfected cells showing the presence of MMTV DNA and the expression of viral RNA were grown near confluency and exposed to 0.7 μM dexamethasone for 24 h, and culture medium was harvested and centrifuged at 1000*g* for 30 min. The supernatant was passaged through a 0.2-μm pore-size filter to remove cellular debris, mixed with 0.5 vol of polyethylene glycol (PEG) 6000 (25% PEG 6000, 1.5 M NaCl) at 4°C for 2 h, and centrifuged again at 12,000*g* for 20 min at 4°C. RNA was extracted from the virus pellet using the TRIzol reagent (Gibco/BRL) and treated with 6 U of RQ DNase (Promega, Madison, WI) for 90 min at 37°C. The DNase was inactivated with an equal volume of 50 mM EDTA, and the RNA was reextracted once with phenol–chloroform, precipitated with ethanol, and resuspended in 30 μl of diethyl pyrocarbonate-treated water.

RT-PCR

RT-PCR was done on the second set of LTR primers. The reactions were carried out with 5 μl of DNase-treated RNA. No amplification of any specific DNA band was seen. Viral cDNA was made from 25 μl total RNA

using the second set LTR reverse primer. Each 50-μl reverse transcription reaction mixture contained 50 mM Tris, 75 mM KCl, 3 mM $MgCl_2$, 600 μM dithiothreitol, 150 μM of each deoxynucleotide triphosphate, 66.4 μM LTR primer, 25 U of RNAguard (Pharmacia Biotech), and 130 U of Moloney murine leukemia virus reverse transcriptage (Gibco/BRL). Second-strand synthesis was carried out with 5 μl of cDNA with the LTR primers described above.

In vivo virus infection assay

The usual test for the infectivity of MMTV in mice is performed by intraperitoneal inoculation of concentrated viral preparations. Shackleford and Varmus (1988) have shown that MMTV-producing rat cells can also be inoculated into mice to establish virus infection. In the present study, JYG-MMTV-producing rat cells were grown in the presence of 0.7 μM dexamethasone for 24 h, resuspended in 1 ml (10^7 cells) of phosphate-buffered saline containing 5 μM dexamethasone, and injected into female endogenous MMTV-free C58/J × CBA/CaJ mice subcutaneously and intraperitoneally. The injections were repeated three times over a period of 2 weeks, and the mice were then bred. The first-litter pups of these rat-cell-injected mice were discarded to eliminate the possibility of MMTV-infected rat cells being transported to the offspring. Pups from the second litter were used for breeding.

Southern hybridization

The expression of MMTV RNA in cultured cells and in various mouse tissues was detected by Southern hybridization. Viral cDNA was made from total RNA; the DNA-PCR was carried out by using 5 μl cDNA from the 50-μl RT reaction. The PCR products were separated on a 1.0% agarose gel, blotted, and hybridized with a 430-bp ^{32}P-labeled LTR probe. The probe (LTR position: 470 to 902 bp) was amplified by PCR, using the forward primer 5′(470)-CAGATTCAGAGGTTAGAAATGGG(493)-3′ and the reverse primer 5′(902)-AGCTCCCTCTTCTGTATAATC(981)-3′. This internal fragment was chosen as a probe to avoid hybridization to the primers used for the DNA- and RT-PCR reactions.

Northern hybridization

Mammary glands obtained from control and experimental lactating mice were analyzed for the presence of MMTV genomic and subgenomic mRNAs following a procedure similar to that which we described previously (Li *et al.,* 1994). Briefly, RNA was extracted with 6 M urea–3 M LiCl and denatured at 65°C for 10 min in a solution containing 50% (w/w) formamide, 2.2 M formaldehyde, and RNA electrophoresis buffer consisting of 20 mM Mops (morpholinepropanesulfonic acid, pH 7.0), 5 mM sodium acetate, and 1 mM EDTA. The RNA samples (20 μg/lane) were then electrophoresed in 1% agarose gel (buffer: 2000 mM Mops, 500 mM NaOAc, 100 mM EDTA, 4% formaldehyde), transferred to a Zeta probe filter in the presence of 20× SSC, and UV cross-linked. The filter was then hybridized with approximately 5×10^7 cpm/ml of a radioactive full-length MMTV probe for 24 h at 65°C in a solution containing 0.2 M Na_2HPO_4, 1 mM EDTA, 1% BSA, 7% SDS, and 16% formamide. The filter was washed three times, 25 min each, in a solution of 0.4 M Na_2HPO_4, 5% SDS, and 0.001 mM EDTA at 65°C and exposed to Kodak X-ray film at −80°C. The radioactive probe was then stripped (dehybridized) from the filter and rehybridized with a chicken β-actin probe to ensure that equivalent amounts of RNA from control and experimental samples were loaded on each lane of the gel.

ACKNOWLEDGMENTS

This work was supported by grants from NIH to N.H.S. (CA-45156) and B.T.H. (R37AI14910). L.X. was supported by the Graduate School of Medicine, Medical College of Georgia, and by an AIDS Basic Research Training Grant from NIH (T32 AI-07389). Thanks are given to B. S. McClellan for technical help, Brenda Newman for art work, and to Rhea-Beth Markowitz for editorial assistance.

REFERENCES

Acha-Orbea, H., Shakhov, A. N., Scrapellino, L., Kolb, E., Muller, V., Vessaz-Shaw, A., Fuchs, R., Blochlinger, K., Rollini, P., Billote, J., Sarafidou, M., MacDonald, H. R., and Diggelmann, H. (1991). Clonal deletion of Vβ14 positive T cells in mammary tumor virus transgenic mice. *Nature* (*London*) **350,** 207–211.

Bittner, J. J. (1936). Some possible effects of nursing on the mammary tumor incidence in mice. *Science* **84,** 162–169.

Brookes, S., Placzek, M., Moore, R., Dixon, M., Dickson, C., and Peters, G. (1986). Insertion elements and transitions in cloned mouse mammary tumor virus DNA: Further delineation of the poison sequences. *Nucleic Acids Res.* **14,** 8231–8245.

Choi, Y., Kappler, J. W., and Marrack, P. (1991). A superantigen encoded in the open reading frame of 3′ long terminal repeat of mouse mammary tumor virus. *Nature* (*London*) **350,** 203–207.

Dickson, C., Smith, R., Brookes, S., and Peters, G. (1984). Tumorigenesis by mouse mammary tumor virus: Proviral activation of a cellular gene in the common integration region *int-2. Cell* **37,** 529–536.

Donehower, L. A., Haung, A. L., and Hager, G. L. (1981). Regulatory and coding potential of the mouse mammary tumor virus long terminal redundancy. *J. Virol.* **37,** 226–238.

Dzuris, J. L., Golovkina, T. V., and Ross, S. R. (1997). Both T and B cells shed infectious mouse mammary tumor virus. *J. Virol.* **71,** 6044–6048.

Evans, G. A., Margulies, D. H., Skykind, B., Seidman, J. G., and Ozato, K. (1982). Exon shuffling: Mapping polymorphic determinants on hybrid mouse transplantation antigen. *Nature* **300,** 755–757.

Fasel, N., Buetti, E., Firzlaff, J., Pearson, K., and Diggelman, H. (1983). Nucleotide sequence of the 5′ noncoding region and part of the *gag* gene of mouse mammary tumor virus: Identification of the 5′ splicing site for subgenomic mRNAs. *Nucleic Acids Res.* **11,** 6943–6955.

Frankel, W. N., Rudy, C., Coffin, J. C., and Huber, B. T. (1991). Linkage of *Mls* genes to endogenous mouse mammary tumor viruses of inbred mice. *Nature* **349,** 526–528.

Gallahan, D., and Callahan, R. (1987). Mammary tumorigenesis in feral mice: Identification of a new *int* locus in mouse mammary tumor virus (Czech II)-induced mammary tumors. *J. Virol.* **61,** 66–74.

Golovkina, T. V., Chernovsky, A., Dudley, J. P., and Ross, S. R. (1992). Transgenic mouse mammary tumor virus superantigen expression prevents viral infection. *J. Virol.* **69,** 637–645.

Golovkina, T. V., Piazzon, I., Nepomnaschy, I., Buggiano, V., de Olanovela, M., and Ross, S. R. (1997). Generation of tumorigenic milk-borne mouse mammary tumor virus by recombination between endogenous and exogeneous viruses. *J. Virol.* **71,** 3895–3903.

Groner, B., Buetti, E., Diggelmann, H., and Hynes, N. H. (1980). Characterization of endogenous and exogenous mouse mammary tumor virus proviral DNA with site-specific molecular clones. *J. Virol.* **36,** 734–745.

Held, W., Shakhov, A. N., Waanders, G. A., Scarpellino, L., Luethy, R., Kraehenbuhl, J. P., MacDonald, H. R., and Acha-Orbea, H. (1992). An exogenous mouse mammary tumor virus with properties of Mls-1 (Mtv-7). *J. Exp. Med.* **175,** 1623–1633.

Held, W., Waanders, G. A., Shakhov, A. N., Scarpellino, L., Acha-Orbea, H., and MacDonald, H. R. (1993). Superantigen induced immune stimulation amplifies mouse mammary tumor virus infection and allows virus transmission. *Cell* **74,** 529–540.

Imai, S., Okumoto, M., Iwai, M., Haga, S., Mori, N., Miyashita, N., Moriwaki, K., Hilgers, J., and Sarkar, N. H. (1994). Distribution of mouse mammary tumor virus in Asian wild mice. *J. Virol.* **68,** 3437–3442.

Kozak, C., Peters, G., Pauley, R., Morris, V., Michalides, R., Dudley, J., Green, M., Davisson, M., Prakash, O., Vaidya, A., Hilgers, J., Verstraeten, A., Hynes, N., Diggelmann, H., Peterson, D., Cohen, J. C., Dickson, C., Sarkar, N., Nusse, N., Varmus, H., and Callahan, R. (1987). A standardized nomenclature for endogenous mouse mammary tumor viruses. *J. Virol.* **61,** 1651–1654.

Li, H. W., Zhao, W., and Sarkar, N. H. (1994). Dietary regulation of mammary tumorigenesis in RIII/Sa mice: Investigation of a possible mechanism. *Cancer Letters* **79,** 199–211.

Majors, J. E., and Varmus, H. E. (1981). Nucleotide sequencing at host-proviral junctions for mouse mammary tumor virus. *Nature* **289,** 253–258.

Majors, J. E., and Varmus, H. E. (1983). Nucleotide sequencing of an apparent proviral copy of *env* mRNA defines determinants of expression of the mouse mammary tumor virus *env* gene. *J. Virol.* **47,** 495–504.

Morris, D. W., Bradshaw, H. D., Billy, H. T., Munn, R., and Cardiff, R. D. (1989). Isolation of a pathogenic clone of mouse mammary tumor virus. *J. Virol.* **63,** 148–158.

Nusse, R., and Varmus, H. E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* **31,** 99–109.

Nusse, R., van Ooyen, D., Cox, Y. K., Fung, T., and Varmus, H. E. (1984). Mode of proviral activation of a putative mammary oncogene (*Wnt*-1) on mouse chromosome 15. *Nature* **307,** 131–136.

Nusse, R., and Varmus, H. E. (1992). *Wnt* genes. *Cell* **69,** 1073–1087.

Ostrowski, M., Haung, A. L., Kessel, M., Wolford, R. G., and Hager, G. L. (1984). Modulation of enhance activity by the hormone responsive regulatory element from mouse mammary tumor virus. *EMBO J.* **3,** 1891–1899.

Peters, G., Brookes, S., Smith, R., and Dickson, C. (1983). Tumorigenesis by mouse mammary tumor virus: Evidence for a common region for provirus integration in mammary tumors. *Cell* **33,** 369–377.

Peterson, D. O., Kriz, K. G., Marich, J. E., and Toohey, M. G. (1985). Sequence organization and molecular cloning of mouse mammary tumor virus DNA endogenous to C57BL/6 mice. *J. Virol.* **54,** 521–531.

Robbins, J., Blondel, B. J., Gallahan, D., and Callahan, R. (1992). Mouse mammary tumor gene *int-3:* A member of the notch gene family transforms mammary epithelial cells. *J. Virol.* **66,** 2594–2599.

Salmons, B., Groner, B. Callberg-Bacq, C. M., and Ponta, H. (1985). Production of mouse mammary tumor virus upon transfection of a recombinant proviral DNA into cultured cells. *Virology* **144,** 101–114.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sarkar, N. H., Haga, S., Lehner, A. S., Zhao, W., Imai, S., and Moriwaki, K. (1994). MMTV proviral integration in and insertional mutation of *Wnt*-oncogenes in the mammary tumors of Chinese wild mice. *Virology* **203,** 52–56.

Saas, B., and Dunn, T. B. (1979). Classification of mouse mammary tumors in Dunn's miscellaneous group including recently reported types. *J. Natl. Cancer Inst.* **62,** 1287–1293.

Scherer, M. T., Ignatowicz, L., Pullen, A., Kappler, J., and Marrack, P. (1997). The use of mammary tumor virus (*Mtv*)- negative and single-*Mtv* mice to evaluate the effects of endogenous viral superantigens on the T cell repertoire. *J. Exp. Med.* **182,** 1493–1504.

Sen, G. C., Haspel, H., and Sarkar, N. H. (1980). Presence of a proteolytic activity in murine mammary tumor virus. *J. Biol. Chem.* **255,** 7098–7101.

Sen, G. C., Smith, S. W., Marcus, S. L., and Sarkar, N. H. (1979). Identification of the messenger RNAs coding for the gag and env products of the murine mammary tumor virus. *Proc. Natl. Acad. Sci. USA* **76,** 1736–1740.

Shackleford, G., and Varmus, H. E. (1988). Construction of a clonable, infectious, and tumorigenic mouse mammary tumor virus and a derivative genetic vector. *Proc. Natl. Acad. Sci. USA* **85,** 9655–9659.

Short, J. M., Fernandez, J. M., Sorge, J. A., and Huge, W. D. (1988). λZap: A bacteriophage λ expression vector with in vivo excision properties. *Nucleic Acids Res.* **16,** 7583–7600.

Ucker, D. S., Ross, S. R., and Yamamoto, K. R. (1981). Mammary tumor virus DNA contains sequences required for its hormone-regulated transcription. *Cell* **27,** 257–266.

Wheeler, D. A., Butel, J. S., Medina, D., Cardiff, R. D., and Hager, G. L. (1983). Transcription of mouse mammary tumor virus: Identification of a candidate mRNA for the long terminal repeat gene product. *J. Virol.* **46,** 42–49.

Wrona, T. J., Lozana, M., and Dudley, J. P. (1998). Mutational and functional analysis of the C-terminal region of the C3H mouse mammary tumor virus. *J. Virol.* **72,** 4746–4755.

Van Nie, R., and Dux, A. (1971). Biological and morphological characteristics of mammary tumors in GR mice. *J. Natl. Cancer Inst.* **46,** 885–898.

Van Nie, R. (1981). Mammary tumors in the GR mouse strain. *In* "Mammary Tumors in the Mouse" (J. Hilgers and M. Slyser, Eds.), pp. 202–266. Elsevier/North-Holland Biomedical Press, Amsterdam.

van Ooyen, A. J., Michalides, R. J., and Nusse, R. (1983). Structural analysis of a 1.7 kilobase mouse mammary tumor virus-specific RNA. *J. Virol.* **46,** 362–370.

Xu, L., Haga, S., Imai, S., and Sarkar, N. H. (1994). Cloning in plasmid of an MMTV from a wild Chinese mouse: Sequencing of the viral LTR. *Virus Res.* **33,** 167–178.